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14. ABSTRACT This project ultimately aims to identify the role of inhibin alpha subunit (INHA) in advanced prostate cancer (PCa). The hypothesis to be tested is that INHA is tumor promoting and pro-metastatic in advanced PCa. To date, we have made significant progress towards understanding the role of INHA in advanced prostate disease. We have demonstrated that increased INHA expression in highly aggressive and metastatic PCa cell line, PC3, further promotes its tumor growth and metastatic ability. Increase in metastasis was further evident by increase lymph vessel density and lymphatic invasion by the cancer cells. This was also accompanied by increase in VEGF-A and VEGF-C expression. Analysis of the clinical specimens demonstrated that INHA expression cannot be used to determine lymph node status in PCa patients. However, the results showed increased INHA expression in normal epithelium and intraepithelial neoplasia regions of the tissues from patient with lymph node metastasis compared to those with organ-confined disease suggesting that INHA may play an important role in promoting the spread of cancer cells to the lymph nodes. We will soon begin our work on understanding the mechanism through which INHA promotes tumor growth and metastasis. Whatever the outcomes of these experiments are, we are sure to contribute significantly to our understanding of the role of INHA in the process of prostate carcinogenesis.					
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## INTRODUCTION

This project ultimately aims to identify the role of inhibin- $\alpha$  subunit (INHA) in prostate carcinogenesis. To date the role of INHA in reproductive cancers is equivocal. This project seeks to test the hypothesis that increased expression of INHA in advanced prostate cancer (PCa) promotes tumor growth and the spread of cancer cells to the lymph nodes. If this hypothesis is proven, then INHA will be implicated as being one of the factors inducing metastatic disease. In addition, we will provide the biological mechanisms affected by increased INHA expression in prostate carcinogenesis. Clinical specimens will also be used to determine INHA expression and lymph node status in PCa patients. To date, we have made significant progress towards demonstrating that increased INHA expression promotes tumor growth and metastasis in advanced PCa, as discussed below.

*Please note: Officially, this annual report covers research from the period 01/02/07 – 31/01/08. However, please note that the principal investigator, Preetika Balanathan has been on full maternity leave since 23/07/07 – 01/01/08. And only worked 2 days per week for the month of January 2008. Therefore the current report covers worked perform from 01/02/07 – 22/07/07.*

## BODY

Task 1: To investigate the tumor promoting and pro-metastatic role of INHA using *in vitro* and *in vivo* models (Months 1-6).

- a. Immunohistochemistry for human mitochondria on tissues (primary prostate tumors and lymph nodes (LNs)) harvested from study already completed will show the presence of human cells in the primary and secondary tumors.

We have completed the aims of Task 1a during the first six months of the project. Specifically, this involved using immunohistochemistry to show presence of human cells in the harvested tissues thereby validating our preliminary observations; *in vivo* data from INHA over-expressing cells showed increased tumor size following orthotopic injection and a 3.5 fold increase (75% versus 20%) in the incidence of metastasis from the primary tumor to surrounding LNs compared to controls. Monoclonal human mitochondria antibody was used to determine the presence of human cells in the tumors (primary prostate tumors and LNs). We also used monoclonal R1 antibody to determine INHA expression in tumors. For detailed description of the methodology see Appendix 1.

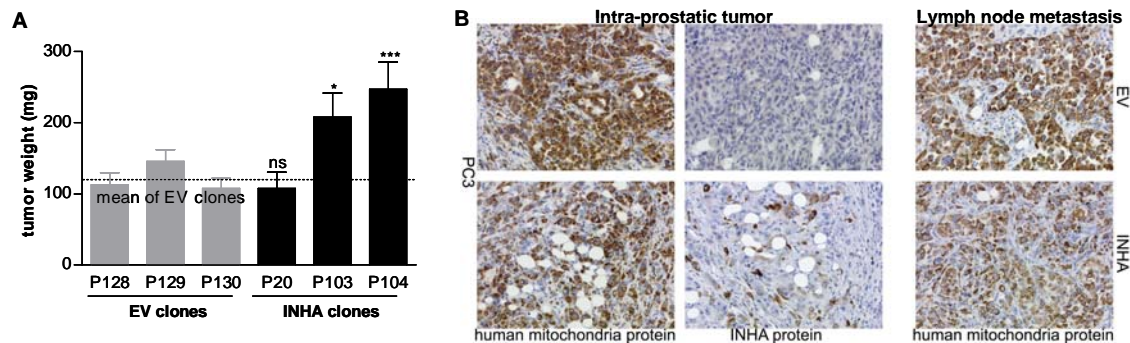
The results showed that INHA over-expression in PC3 cells had no effect on orthotopic tumor take but a significant increase ( $p = 0.0341$ ) in the incidence of LN tumors was observed (Table 1). INHA-transfected PC3 clones generally produced significantly larger tumors (Fig. 1a) when compared to the mean of their respective empty vector (EV) clones. Positive immunostaining for human mitochondrial protein confirmed that the primary and metastatic lesions originated from intra-prostatic injection of human cells.

INHA immunostaining was used to confirm INHA expression in prostate tissues injected with INHA transfected clones (Fig. 1b).

**Table 1 Metastases from intra-prostatic inoculations of PC3 clones**

Pooled cell line	tumor take	lymph node metastases
empty vector clones	25/25 (100%)	5/25 (20%)
INHA clones	20/20 (100%)	15/20 (75%)*

\*  $p = 0.0341$

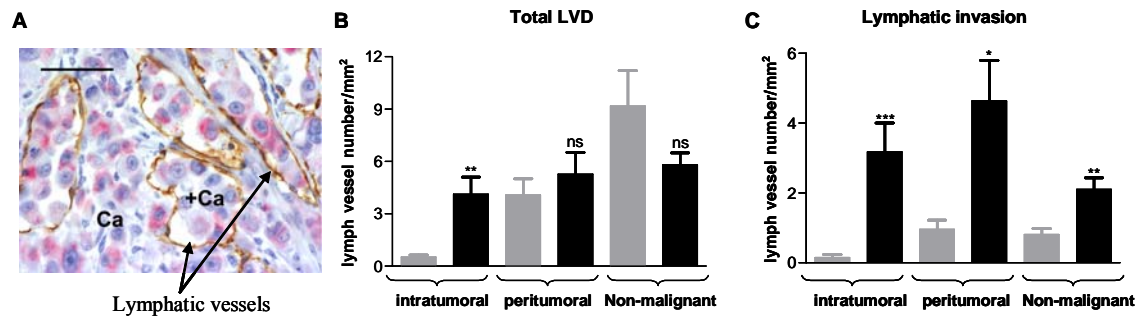


**Figure 1 Effect of INHA over-expression on tumor growth and metastasis.** A, Tumor weights are shown for PC3 prostate tumors. Over-expression of INHA in tumor cells increases tumor growth of PC3 cells following orthotopic inoculation. ns, not significant; \*  $p$  0.01 – 0.05, \*\*\*  $p$  < 0.001 significant difference between the mean of the EV clones (mean for PC3 EV clones is 141.5) and individual INHA-transfected clones. Dotted line denotes the mean for EV clones. Data shown as mean  $\pm$  standard error of the mean. B, Immunohistochemistry of the prostate and LN tumors using human mitochondria staining confirmed the human origin of the cells in PC3 inoculated mice. INHA expression in the intra-prostatic tumors was also confirmed. Bar 100 $\mu$ m.

- b. Immunohistochemistry for human mitochondria and LYVE-1 on the primary prostate tumors will determine lymph vessel density (LVD) in the intratumoral, peritumoral and normal regions in the tissues.

Changes to LVD and lymphangiogenesis are often associated with metastatic spread of cancer cells to the LNs (1, 2). To understand the mechanisms and to provide proof of metastatic spread observed in the mice injected with INHA-positive cells we stained PC3 INHA and EV orthotopic tumors for LYVE-1, and human mitochondrial antibody to determine LVD and the degree of invasion of tumor cells into lymphatic vessels (lymphatic invasion) in the tissues (Fig. 2a). Stereological analysis of these tumors revealed a significant increase ( $p = 0.0023$ ) in the LVD in the intratumoral regions with no difference in LVD in peritumoral and surrounding non-malignant regions of INHA-positive tumors compared to the controls (Fig. 2b). Data also revealed significant increase in lymphatic invasion in the intratumoral ( $p = 0.0002$ ), peritumoral ( $p = 0.0225$ ) and non-

malignant ( $p = 0.0077$ ) regions of the tissue in INHA-positive tumors compared to the controls (Fig. 2c). For detailed description of the methodology see Appendix 1.



**Figure 2 Increase in lymphatic vessel density and lymphatic invasion in PC3 tumors.** A, Lymphatic vessels (LVs) were stained with LYVE-1 antibody (brown) and human prostate cells (Ca) with human mitochondria antibody (purple). Bar 50 $\mu$ m. The total number of LVs (B) and LVs with cancer cells in their lumen (C) (for example of such a vessel see “+Ca” in panel A) in the intratumoral, peritumoral and non-malignant (benign region adjacent to the tumor) regions of the primary prostate tumor were counted. \*  $p$  0.01 – 0.05, \*\*  $p$  0.001 – 0.01, \*\*\*  $p$  < 0.001 and no significant (ns) difference between LVD in INHA over-expressing primary tumor compared to EV tumors. The bars represent: EV-transfected PC3 clones in grey, INHA-transfected clones in black. Data shown as mean  $\pm$  standard error of the mean.

- c. Collection of fresh prostate tissues from 3 prostate cancer patients, isolation of lymphatic endothelial cells (LECs) from and culturing them in the presence of PCa cells with and without INHA expression and/ or recombinant inhibin protein will determine the effect of the cancer cells and recombinant inhibin protein on LEC tube number and length.

Before this work begins, we were required to obtain Human Ethics approval to access human prostate tissues from patients undergoing radical prostatectomy surgery. Our laboratory already holds a human ethics approval at East Epworth Hospital, Boxhill, Melbourne, Australia to obtain fresh prostate tissues from patient undergoing surgery for another project “Role of tumor stroma in prostate carcinogenesis”. The original application was amendment to include access of tissues for isolating human lymphatic endothelial cells. The primary approval was granted from Epworth Human Ethics committee (Approval Number: 34306 on 06 December 2006 [see appendix 2]. Secondary approval was granted from Monash University Standing Committee on Ethics in Research Involving Humans (Approval Number: 2004/145MC) on 13 June 2007 [see appendix 3], which was necessary since some staff involved in the project are employees of Monash University.

There has been no other progress made for this aim at the present time.

Task 2: To determine the mechanism through which INHA may promote tumor growth and metastasis (Months 3 – 24).

- a. ELISAs for VEGF-C and VEGF-D expression at the protein level will confirm changes in INHA over-expressing PC3 cells and empty vector (EV) transfected PC3 cells.

We have completed the aims of Task 2a during the first six months of the project. The observed increase in LVD in INHA-positive PC3 tumors suggested that the metastatic spread of the cancer cells from the primary tumor site to the LNs occurs through the process of lymphangiogenesis. Members of the vascular endothelial growth factor (VEGF) family, VEGF-C, VEGF-D and more recently VEGF-A, have been associated with lymphangiogenesis, mediating their effects through vascular endothelial growth factor receptors 2 and 3 (VEGF R2 and VEGF R3) (3-5). Our preliminary data showed that there was no change in VEGF-D mRNA levels in INHA over-expressing PC3 cells compared their EV controls, therefore it was decided not to analyze VEGF-D expression any further. However, we went on to determine the expression of VEGF-A and VEGF-C protein by ELISA, in INHA- and EV-transfected clones *in vitro*. VEGF-A mRNA levels were also determined. For detailed description of the methodology see Appendix 1.

VEGF-A ( $p = 0.0002$ ) and VEGF-C ( $p = < 0.0001$ ) mRNA levels were significantly increased in INHA over-expressing PC3 cells (Table 2). Secreted VEGF-C protein levels were significantly increased ( $p = 0.0011$ ) in the INHA over-expressing PC3 clones compared to their EV clones, however there was no significant change in secreted VEGF-A levels (Table 2).

**Table 2 Effect of over-expressing INHA on VEGF-A and VEGF-C**

	PC3	
	EV clones	INHA clones
<b>VEGF-A</b>		
normalised mRNA	0.016 ± 0.002	0.0423 ± 0.006***
protein (µg/µl)		
cell lysate	626.8 ± 25.04	500.9 ± 24.51*
conditioned media	1794 ± 40.31	1712 ± 34.64
<b>VEGF-C</b>		
normalised mRNA	12.76 ± 1.59	34.96 ± 2.76***
protein (µg/µl)		
cell lysate	319.6 ± 49.21	656.7 ± 46.21**
conditioned media	3377 ± 566.0	6892 ± 531.6**

\*  $p = 0.01 - 0.05$

\*\*  $p = 0.01 - 0.001$

\*\*\*  $p = < 0.001$

- b. Microarray technology, siRNA and small inhibiting molecules will comprehensively analyze and identify the pathway(s) inhibin affects in regulating its tumor promoting and pro-metastatic role.

There has been no progress towards this aim at present.

- c. RNA interference technology via a viral delivery system will be used to down-regulate INHA and VEGF-C expression in INHA over-expressing cells and orthotopic inoculation of these cells in mice will show and confirm that INHA stimulated VEGF-C is responsible for increased in LVD and increased incidence of LN metastasis. We project to use 100 male SCID mice for this study (10 mice per group).

There has been no progress towards this aim at present with regards down-regulating INHA and VEGF-C expression in INHA over-expressing cells. However, before studies on mice can begin, we were required to obtain Animal Ethics approval from Monash Medical Centre Animal Ethics Committee to use mice for this project. The approval was granted (Application number: MMCA 2006/45) on 20 March 2007 [see appendix 4].

- d. Orthotopic inoculation of INHA and empty vector (EV) transfected PC3 cells followed regular injections of neutralizing antibodies will be used to block VEGF receptor signaling in the cells. This will show that by blocking VEGF receptor signaling we can reduce LVD and the incidence of LN metastasis. We project to use 60 male SCID mice for this study (10 mice per group).

There has been a delay towards this aim since we were unable to obtain the neutralizing antibodies (VEGF R2 and VEGF R3) to block VEGF receptor signaling in the cells as originally planned. To minimize this having an effect on this project and delaying the experiments further we formed a collaborative research with Schering AG, Corporate Research Oncology who has sent us PTK/ZK, a compound known to specifically block VEGF receptor signaling (6). PTK/ZK has successfully been used in both animal experiments (6) and human clinical trial for advanced colorectal cancer, acute myeloid leukemia and liver metastases (7, 8).

Before this work begins, we were required to obtain Animal Ethics approval from Monash Medical Centre Animal Ethics Committee to use mice for this project. The approval was grants (Application number: MMCA 2006/45)) on 20 March 2007 [see appendix 4]. The use of this compound instead of the neutralizing antibodies also reduces the number of mice to be used for this aim. We will use 40 male SCID mice for this study (10 mice per group).

There has been no further progress towards this aim at present.



Task 3: To determine the utility of INHA for the diagnosis of patients with highly aggressive and/ or metastatic PCa (Months 3-8)

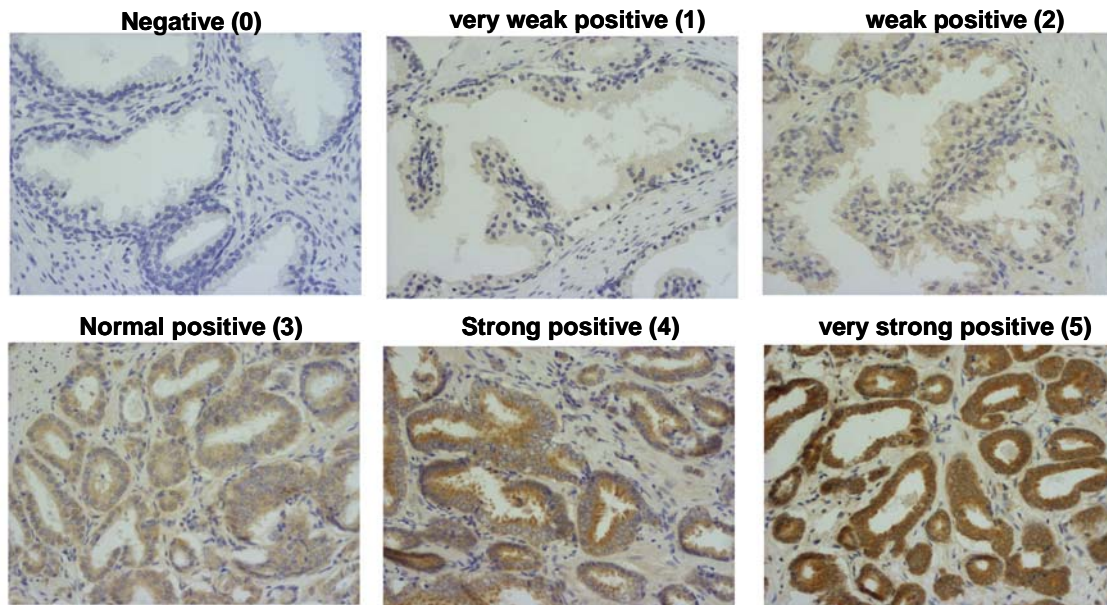
- a. Collection of archival human prostate tissue of at least 50 men who under radical prostatectomy. Tissues will be collected from patient who had organ-confined disease and those with lymph node metastasis.

We have completed the aims of Task 3a during the first six months of the project. Before this work begins, we were required to obtain Human Ethics approval to access archival human prostate tissues from patients who under went radical prostatectomy surgery. The approval was granted from Monash University Standing Committee on Ethics in Research Involving Humans (Approval Number: CF07/0854 – 2007/0223HT) on 16 May 2007 [see appendix 5]. We collected archival tissues from patients who had organ-confined disease and those with lymph node metastasis.

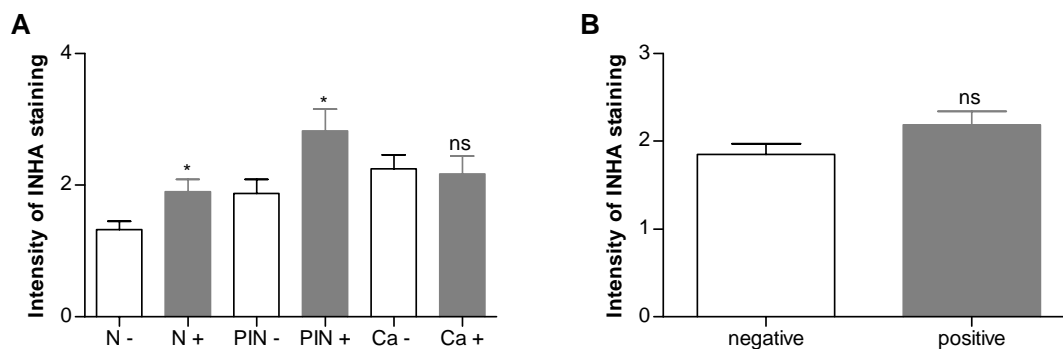
- b. Immunohistochemistry for INHA, VEGF-C and D2-40 will show the utility of INHA as a diagnostic or prognostic marker for PCa patients.

We have completed the aims of Task 3b during the first six months of the project. To reduce wastage of precious human prostate tissues it was decided to use a cohort of patient tissues that have already being evaluated for clinicopathological characteristics, VEGF-C expression and LVD (using D2-40) (1, 9) by our collaborating investigator; Elizabeth Williams. A number of independent studies have shown increases in INHA expression to be associated with PCa progression (10, 11). To determine if INHA expression can be correlated to lymph node status we used tissues from a cohort of PCa patients who had organ-confined disease and those who had lymph node metastasis. We obtained 20 radical prostatectomy specimens were from patients with organ-confined disease, while the remaining 16 specimens were from patients with LN metastases. The PO#12 antibody was used to determine the expression pattern of INHA in the prostate tissues. For detailed description of methodology see Appendix 1. An example of the INHA staining and scoring is provided in Figure 3.

Immunostaining revealed significant increase in INHA staining in normal epithelial and PIN regions with no change in the staining in the cancer regions of the prostate tumor tissues in patients with LN metastasis compared to patients with organ-confined disease (Fig 4A). However, there was no significant change in overall INHA expression in patients with LN metastasis compared to patients with organ-confined disease (Fig 4B).



**Figure 3 Example of immunohistochemistry staining intensity used to evaluate the intensity of INHA staining in the prostate tissues.** Each immunostained tissue section was assessed and staining intensity in the different regions and grades of tumor was scored as following: -: negative (0); +/-: very weak positive staining (0.5); +: weak positive staining (1); ++: normal positive staining (2); +++: strong positive staining (3); ++++: very strong positive staining (4). Intensity of INHA staining in normal epithelial and intraepithelial neoplasia (PIN) and cancer regions (Gleason grade G1-G5) were analyzed to determine the pattern of INHA expression in the prostate tissues from patients with organ-confined disease and those with LN metastasis.



**Figure 4 INHA staining in PCa patients with organ confined and metastatic disease.** A, INHA immunostaining intensity in normal epithelial (N), PIN and cancer regions (Ca) was compared in patients with organ-confined prostate cancer (-) and those with metastasis to the lymph nodes (+). \* p 0.01 – 0.05 and no significant (ns) difference between the respective regions in organ confined and metastatic disease. B. Overall, there was no significant difference in INHA intensity in tissues from patients with organ-confined prostate cancer (negative) and those with metastasis to the LN (positive).

### KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that over-expression of INHA enhanced metastatic ability of tumor cells *in vivo*.
- Determined VEGF-A and VEGF-C expression at the protein level in INHA over-expression PC3 cells and the controls.
- Gained Human Ethics approval for collection of fresh and archival human prostate tissues.
- Obtained PTK/ZK, a compound which blocks VEGF family member signaling by blocking their receptor activity.
- Determined INHA expression pattern in prostate cancer patients with organ-confined and metastatic disease.

### REPORTABLE OUTCOMES

	<i>Reportable outcomes that have resulted from this research:</i>
Manuscripts	Submitted to Oncology. Due to the copyright issues only the title page, abstract and methodology used in the aims of this project is provided at this stage [see appendix 1]
Abstracts and presentations	<p><u>Preetika Balanathan</u>, Elizabeth D Williams, Hong Wang, Marc G Achen, Steven A Stacker, Gail Risbridger (2008) <b>Shift in the tumor suppressive activity of inhibin-<math>\alpha</math> subunit during the transition from androgen-dependent to androgen-independent prostate cancer</b> TGF<math>\beta</math> family in Homeostasis and Disease – Keystone Symposia, Santa Fe New Mexico, USA (poster presentation). [see appendix 6]</p> <p><u>Preetika Balanathan</u>, Elizabeth D Williams, Hong Wang, Marc G Achen, Steven A Stacker, Gail Risbridger (2008) <b>Tumor suppressive activity of inhibin-<math>\alpha</math> subunit is altered during the transition from androgen-dependent to androgen-independent prostate cancer</b> Lorne Cancer, Victoria, Australia (poster presentation). [see appendix 7]</p>
Patents and licenses applied for and/or issued	Nil
Degrees obtained that are supported by this award	Nil
Development of cell lines, tissues or serum repositories	Nil

Informatics such as databases and animal models, etc	Nil
Funding applied for based on work supported by this award	GlaxoSmithKline (GSK) Post Graduate Support Grant for 2008 [see appendix 8]  ANZ Philanthropy Trust Fund Grant 2008 [see appendix 9]  Keystone Symposia – Travel scholarship [see appendix 10]
Employment or research opportunities applied for and/or received based on experience/training supported by this award	Nil

### CONCLUSIONS

In summary, we have made significant progress towards understanding the role of INHA in advanced PCa. We have demonstrated increased tumor size and increased metastasis to the LNs by INHA over-expressing PC3 cells compared to the controls. The increase in metastasis was further evident by increase in total LVD and lymphatic invasion which was accompanied by increase in VEGF-C expression.

Using clinical specimens we have been able to determine that there is evidence of increased INHA expression in normal epithelium and PIN regions (pre-cancerous lesions) of patients with metastatic disease compared to those with organ-confined disease. Although the current results showed that INHA expression cannot be used to determine LN status in PCa patients, it does suggest that the increase in INHA expression by normal epithelium and PIN regions of the prostate in patients with LN metastasis may have a paracrine role that, directly or indirectly, promotes the spread of cancer cells from the primary prostate tumor to the LNs.

We will soon begin our work on understanding the mechanism through which INHA promotes tumor growth and metastasis. Whatever the outcomes of these experiments are, we are sure to contribute significantly to our understanding of the role of INHA in the process of prostate carcinogenesis.

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## APPENDIX 1 - MANUSCRIPT

### **Tumor suppressive activity of inhibin- $\alpha$ subunit is altered during prostate cancer progression**

Preetika Balanathan<sup>1</sup>, Elizabeth D Williams<sup>2</sup>, Hong Wang<sup>1</sup>, John S Pedersen<sup>3</sup>, Lisa G Horvath<sup>4,5</sup>, Marc G Achen<sup>6</sup>, Steven A Stacker<sup>6</sup>, Gail P Risbridger<sup>1</sup>

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*Short title:* Shift in the role of INHA during PCa progression

*Key Words:* inhibin- $\alpha$  subunit, tumor suppressor, pro-metastatic factor, prostate cancer, metastasis

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*Financial Support:* Post-doctoral training award by United States Department of Defense [PB]. Cancer Council Victoria grant-in-aid [EDW]. Cancer Institute of New South Wales [LGH]. Senior Research Fellowship from Australian National Health and Medical Research Council (NH&MRC) and the Pfizer Foundation [MGA and SAS, respectively] and by a NH&MRC program grant. Principal Research Fellowship from Australian NH&MRC [GPR].

**Abstract**

Inhibin- $\alpha$  subunit (INHA) is a member of the Transforming Growth Factor  $\beta$  superfamily and has been an important biological marker for ovarian cancer but its role in prostate cancer (PCa) is unclear. We have observed up- and down-regulation of INHA expression in PCa dependent on the stage of disease and proposed that INHA has tumor suppressive and pro-metastatic activity in different stages of PCa progression. The present study was designed to elucidate the role of INHA in regulation of tumor cell growth, migration and the tumorigenic and metastatic potential of two metastatic human PCa cell lines, LNCaP and PC3, which differ in androgen-responsiveness and growth characteristics. We report here that over-expression of INHA in LNCaP cells reduced cell proliferation, migration and tumor growth supporting its tumor suppressive activity. In contrast, loss of tumor suppressive activity and gain in metastatic properties of INHA was observed in PC3 cells which demonstrated increased cell proliferation, migration, tumor growth and metastasis. Analysis of INHA staining in clinical specimen suggested a potential paracrine role for INHA metastasis to the lymph nodes. Our results demonstrate the different roles of INHA in PCa and provide an explanation for the paradoxical expression of INHA during PCa progression.



**Materials and Methods*****Confirmation of proteins expression by Enzyme-linked immunosorbent assay (ELISA)***

Cell lysates and conditioned media (media conditioned for 24hrs) were prepared from EV- and INHA-transfected clones. Total protein (1  $\mu\text{g}/\mu\text{l}$ ) was used for further analysis. VEGF-A and VEGF-C ELISAs were measured in duplicates using specific ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). Two biological replicates were examined.

***Intra-prostatic inoculation of LNCaP and PC3 cells***

The experiments were conducted with approval of the Monash Medical Centre Animal Ethics Committee and were in accordance with National Health and Medical Research Council (NH&MRC) of Australia Guidelines. Male SCID mice 6-8 weeks of age were purchased from the Australian Resource Centre (Perth, Australia). LNCaP ( $2 \times 10^6$ ) or PC3 ( $5 \times 10^5$ ) clones were injected orthotopically into the ventral lobe of the prostate gland (10 animals/clone) as previously described (1). After 7-9 weeks, mice were euthanased and primary tumors removed and weighed. In addition, regional lymph nodes were also removed for analysis. Monoclonal human mitochondria antibody (1:100; Chemicon, Temecula, CA) was used to determine the presence of human cells in the tumors as previously described (2). Monoclonal R1 antibody (7.5  $\mu\text{g}/\text{ml}$ ), kindly provided by Dr Nigel Groome was used to determine INHA expression in tumors as previously described (3).

***Lymphatic vessel density (LVD) in the intra-prostatic tumors***

Lymphatic vessels were identified using lymphatic vascular endothelial hyaluronan receptor (LYVE-1), a marker of lymphatic endothelium (4). Invasion of tumor cell into lymphatics was monitored by the presence of human mitochondrial protein stained cancer cells in lymph vessels. Double immunostaining for LYVE-1 and mitochondria was performed on a DAKO Autostainer (DAKO, Denmark). The sections were incubated with LYVE-1 antibody (Fitzgerald, MA) diluted at 0.5 µg/ml for 2 hours. LYVE-1 was detected by incubation with Envision polymer-anti-rabbit-HRP (DAKO) for 15min and visualised with diaminobenzidine (DAKO). Sections were then incubated with Double Staining Enhancer (Zymed, San Francisco, CA) for 15min and exposed to mitochondrial antibody (Chemicon) diluted at 1/200 for 2 hours. Secondary antibody, biotinylated rabbit-anti-mouse IgG1 (Zymed) was applied and the immunoreactivity was detected by ExtrAvidin-Alkaline phosphatase (Sigma, USA) and visualized by reaction with Vector-red (Vector Laboratories, CA). The sections were counterstained with Hematoxylin (DAKO) and immunolocalization was examined using an Olympus BX-60 microscope.

Lymphatic vessels were counted using stereological methods as previously described (3). Lymphatic vessels were counted within tissue sections (of randomly selected INHA-positive prostate tumors, n=15 and EV tumors, n=11; using n=2 randomly selected sections per tumor) to assess the LVD within the tumor (intratumoral) region, the region in contact with both the tumor and the stroma (peritumoral) and the surrounding non-malignant regions (benign region adjacent to the tumor). LVD was expressed as the number of lymph vessels per millimetre squared.

***Analysis of clinical material******INHA expression in PCa patients and lymph node status***

This study was conducted with ethics approval of the St. Vincent's Hospital Human Ethics Committee and was in accordance with Australian NH&MRC Guidelines. Archival formalin-fixed paraffin-embedded tissue blocks were retrieved from 36 patients with prostate carcinoma who underwent radical prostatectomy. Briefly, 20 radical prostatectomy specimens were from patients with organ-confined disease, while the remaining 16 specimens were from patients with lymph node metastases. The clinicopathological characteristics, VEGF-C expression and LVD of this cohort have been described previously (5, 6). In the past we used the readily available, well characterized R1 antibody to detect INHA in our INHA over-expressing cells and tumors. However, our laboratory recently demonstrated the utility of another monoclonal INHA antibody, PO#12 for analysis of clinical specimens (7, 8). The PO#12 antibody (kindly provided by Dr Nigel Groome) was used to determine the expression pattern of INHA in the prostate tissues as previously described (7). Time of incubation for the antibody was 120min and the concentrations used was 5 µg/ml. Each immunostained tissue section was assessed and staining intensity in the different regions and grades of tumor was scored as following: -: negative (0); +/-: very weak positive staining (0.5); +: weak positive staining (1); ++: normal positive staining (2); +++: strong positive staining (3); ++++: very strong positive staining (4). The intensity was scored by two experienced research scientists [HW and EDW]. Intensity of INHA staining in normal epithelial and intraepithelial neoplasia (PIN) and cancer regions (Gleason grade G1-G5) were analyzed

to determine the pattern of INHA expression in the prostate tissues from patients with organ-confined disease and those with lymph node metastasis.

Reference:

1. Zeng, Y., Opeskin, K., Goad, J., Williams, E.D. Tumor-Induced Activation of Lymphatic Endothelial Cells via Vascular Endothelial Growth Factor Receptor-2 Is Critical for Prostate Cancer Lymphatic Metastasis. *Cancer Res.* , *66*: 9566-9575, 2006.
2. McCulloch, D. R., Opeskin, K., Thompson, E.W., Williams, E.D. BM18: A novel androgen-dependent human prostate cancer xenograft model derived from a bone metastasis. *Prostate.* , *65*: 35-43, 2005.
3. Balanathan, P., Ball, E.M.A., Wang, H., Harris, S.E., Shelling, A.N., Risbridger, G.P. Epigenetic regulation of inhibin  $\alpha$ -subunit gene in prostate cancer cell lines. *J Mol Endocrinol.*, *32*: 55-67, 2004.
4. Banerji, S., Ni, J., Wang, S. X., Clasperm, S., Su, J., Tammi, R., Jones, M., and Jackson, D. G. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol.* , *144*: 789-801, 1999.
5. Zeng, Y., Opeskin, K., Horvath, L.G, Sutherland, R.L, Williams, E.D. Lymphatic vessel density and lymph node metastasis in prostate cancer. *Prostate.* , *65*: 222-230, 2005.
6. Zeng, Y., Opeskin, K., Baldwin, M. E., Horvath, L. G., Achen, M. G., Stacker, S. A., Sutherland, R. L., and Williams, E. D. Expression of vascular endothelial growth factor receptor-3 by lymphatic endothelial cells is associated with lymph node metastasis in prostate cancer. *Clin Cancer Res.*, *10*: 5137-5144, 2004.
7. Risbridger, G. P., Shibata, A., Ferguson, K.L., Stamey, T.A., McNeal, J.E., Peehl, D.M. Elevated expression of inhibin  $\alpha$  in prostate cancer. *J Urol.*, *171*: 192-196, 2004.
8. Risbridger, G. P., Ball, E.M., Wang, H., Mellor, S.L., Peehl, D.M. Re-evaluation of inhibin  $\alpha$  subunit as a tumour suppressor in prostate cancer. *Mol Cell Endocrinol.*, *225*: 73-76, 2004.

## APPENDIX 2: EPWORTH HREC APPROVAL



27 December 2006

Prof. Gail Risbridger  
**c/o Courtney Bamford**  
Centre for Urological Research  
Monash Institute of Medical Research  
27 Wright Street,  
CLAYTON VIC 3168

Dear Prof. Risbridger,

**Re: ROLE OF TUMOUR STOMA IN PROSTATE CARCINOGENESIS**  
**Epworth Study No 34306**

Thank-you for your requested amendments and correspondence dated the 15<sup>th</sup> of November 2006.

The Epworth Healthcare HREC accepted these amendments at their meeting on the 6<sup>th</sup> of December 2006.

Thank-you for keeping the committee updated on the progress of your study and we look forward to receiving your annual report in due course.

Yours sincerely,

A handwritten signature in black ink, appearing to read "Louise Grey".

Louise Grey  
HREC Coordinator  
Epworth Hospital  
89 Bridge Rd.  
RICHMOND VIC 3121

## APPENDIX 3: SCERH APPROVAL

<https://mail-store-2.its.monash.edu.au/frame.html?rtfPossible=true...>

From [scerh <scerh@adm.monash.edu.au>](mailto:scerh@adm.monash.edu.au)

Sent Wednesday, June 13, 2007 12:59 pm

To [Renea Taylor <Renea.Taylor@med.monash.edu.au>](mailto:Renea.Taylor@med.monash.edu.au), [Gail Risbridger <Gail.Risbridger@med.monash.edu.au>](mailto:Gail.Risbridger@med.monash.edu.au), [Preetika Balanathan <Preetika.Balanathan@med.monash.edu.au>](mailto:Preetika.Balanathan@med.monash.edu.au)

Cc

Bcc

Subject Monash Human Ethics - 2004/145MC: Role of tumor stroma in prostate carcinogenesis - Request for Amendment

PLEASE NOTE: To ensure speedy turnaround time, this correspondence is now being sent by email only. If you would prefer a hard copy on letterhead, please contact the Human Ethics Office (9905 2076 or [scerh@adm.monash.edu.au](mailto:scerh@adm.monash.edu.au)) and a hard copy will be posted to you.

We would be grateful if first-named investigators could ensure that their co-investigators are aware of the content of the correspondence.

Dr Renea Taylor  
Centre for Urological Research  
Faculty of Medicine, Nursing and Health Sciences  
Monash Medical Centre Campus

13 June 2007

2004/145MC: Role of tumor stroma in prostate carcinogenesis

Dear Researchers,

Thank you for submitting further information as requested by the Standing Committee on Ethics in Research Involving Humans (SCERH) with respect to the Request for Amendment to the above named project.

This is to advise that the requested amendments dated 2 April 2007, received in our office on 5 April 2007, have been approved and the project can proceed according to your approval given on 22 April 2004.

Monash SCERH therefore approves your submission approved by the Epworth Human Ethics Committee, for Epworth study 34306, on 6 December 2006.

Thank you for keeping the Committee informed.

Mrs Lyn Johannessen  
Acting Human Ethics Officer (on behalf of SCERH)

Cc: Prof Gail Risbridger, Dr Preetika Balanathan

**APPENDIX 4: MMCA APPROVAL****Memo**

**To:** Prof. Gail Risbridger, MIMR  
**From:** Glenda Johnson  
**Date:** 20 March 2007  
**Subject:** Notice of Approval – Project No. MMCA 2006/45

Are inhibin- $\alpha$  and VEGF-C responsible for spread of cancer cells in our model of advanced prostate cancer?

**Director:**  
Professor Bryan Williams

**Executive Director:**  
Professor Adrian Walker

**Centre Directors:**  
Professor Paul Hertzog  
Professor Stephen Holdsworth  
Dr Michael Holland  
Professor Martin Pera  
Professor Gail Risbridger  
Associate Professor Peter Rogers

**Patrons:**  
Right Hon Sir Zelman Cowen AK, GCMG, GCVO  
Vice Chancellor Professor Richard Larkins AO

Please find attached a copy of the Final Approved proposal.

The project is approved from 01/01/07 to 31/12/2010 subject to the following conditions.

1. An Annual Report must be provided each January.
2. A Final Report is submitted to the MMC Animals Ethics Committee within six months of completion of the project.
3. Unexpected or adverse events, which impact on the welfare of the animals, must be immediately reported to the Chairperson of the AEC.
4. Any changes to location of animal housing or experimental location details are to be forwarded immediately to the AEC.
5. Special Conditions - None
6. Special Responsibilities by Animal House Staff – None
7. Approved Animal Usage is:-

SPECIES	Total No.
Mouse SCID 6-8 week old/ male	140

Please destroy any previous versions of the proposal and replace with the enclosed authorized document.

Regards,

A handwritten signature in black ink, appearing to read 'Glenda Johnson'.

Glenda Johnson  
MMC Animal Ethics Committee 'A'  
MIMR, Level 3  
MMC Clayton  
Tel. 9594-7342  
Email. Glenda.johnson@med.monash.edu.au

## APPENDIX 5: SCERH APPROVAL



Standing Committee on Ethics in Research Involving Humans (SCERH)  
Research Office

Prof Gail Risbridger  
Department of Centre for Urological Research  
Faculty of Medicine, Nursing and Health Sciences  
Monash Medical Centre Campus

16 May 2007

**CF07/0854 - 2007/0223HT: Protein expression patterns in human prostate cancer tissues**

Dear Researchers,

Thank you for the information provided in relation to the above project. The items requiring attention have been resolved to the satisfaction of the Standing Committee on Ethics in Research Involving Humans (SCERH). Accordingly, this research project is approved to proceed.

### Terms of approval

1. This project is approved for five years from the date of this letter and this approval is only valid whilst you hold a position at Monash University.
1. It is the responsibility of the Chief Investigator to ensure that all information that is pending (such as permission letters from organisations) is forwarded to SCERH, if not done already. Research cannot begin at any organisation until SCERH receives a letter of permission from that organisation. You will then receive a letter from SCERH confirming that we have received a letter from each organisation.
2. It is the responsibility of the Chief Investigator to ensure that all investigators are aware of the terms of approval and to ensure the project is conducted as approved by SCERH.
3. You should notify SCERH immediately of any serious or unexpected adverse effects on participants or unforeseen events affecting the ethical acceptability of the project.
4. The Explanatory Statement must be on Monash University letterhead and the Monash University complaints clause must contain your project number.
5. **Amendments to the approved project:** Changes to any aspect of the project require the submission of a Request for Amendment form to SCERH and must not begin without written approval from SCERH. Substantial variations may require a new application.
6. **Future correspondence:** Please quote the project number and project title above in any further correspondence.
7. **Annual reports:** Continued approval of this project is dependent on the submission of an Annual Report. Please provide the Committee with an Annual Report determined by the date of your letter of approval.
8. **Final report:** A Final Report should be provided at the conclusion of the project. SCERH should be notified if the project is discontinued before the expected date of completion.
9. **Monitoring:** Projects may be subject to an audit or any other form of monitoring by SCERH at any time.
10. **Retention and storage of data:** The Chief Investigator is responsible for the storage and retention of original data pertaining to a project for a minimum period of five years.

All forms can be accessed at our website [www.monash.edu.au/research/ethics/human/index.html](http://www.monash.edu.au/research/ethics/human/index.html)

We wish you well with your research.

  
Mrs Lyn Johannessen  
Acting Human Ethics Officer (on behalf of SCERH)

Cc: Dr Preetika Balanathan, Prof Rob Sutherland, Dr Elizabeth Williams



## APPENDIX 6: TGF $\beta$ – KEYSTONE SYMPOSIA ABSTRACT

### Shift in the tumor suppressive activity of inhibin- $\alpha$ subunit during the transition from androgen-dependent to androgen-independent prostate cancer

Preetika Balanathan<sup>1</sup>, Elizabeth D Williams<sup>2</sup>, Hong Wang<sup>1</sup>, Marc G Achen<sup>3</sup>, Steven A Stacker<sup>3</sup>, Gail Risbridger<sup>1</sup>. <sup>1</sup>Centre for Urological Research, <sup>2</sup>Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. <sup>3</sup>Ludwig Institute for Cancer Research, Post Office Box 2008, Royal Melbourne Hospital, Melbourne, Victoria, Australia.

The inhibin field has been perplexed by the information that inhibin- $\alpha$  subunit (INHA), a member of the TGF $\beta$  superfamily is a tumor suppressor in mice yet is elevated in women with ovarian cancer. Similarly we have observed up- and down-regulation of INHA expression in prostate cancer (PCa) dependent on the stage of disease. We proposed that INHA is tumor suppressive in androgen-dependent (AD) stage of the disease but loses its tumor suppressive activity or gains metastatic properties in androgen-independent (AI) stage of the disease. Recently, loss of TGF $\beta$  receptor RIII (TGF $\beta$ RIII), a receptor for inhibin has been proposed to be an explanation for the different activities of INHA in PCa.

We evaluated the functional role of INHA in two well known PCa cell lines which differ in behavior and molecular makeup and have close resemblance to primary prostate disease. The AD, LNCaP and AI PC3 cell lines were stably transfected with cDNA for INHA and evaluated for their sensitivity to INHA expression in the presence of endogenous levels of TGF $\beta$ RIII. Over-expression of INHA in AD LNCaP cells decreased cell proliferation and migration and reduced tumor growth supporting the role of INHA as a tumor suppressor. In contrast, over-expression of INHA in AI PC3 cells increased cell proliferation, migration, tumor growth and metastasis. This supports the loss of tumor suppressive activity or gain in metastatic properties for INHA in AI stage of the disease. The shift in the tumor suppressive activity of INHA was further evident by increase in lymph node metastasis in the INHA over-expressing PC3 tumors which was accompanied by an elevation of lymphatic vessel density and tumor cell invasion into lymphatics. These effects were associated with up-regulation of the lymphangiogenic growth factor, VEGF-C. Consistent with other studies our work revealed that LNCaP cells expressed significantly more TGF $\beta$ RIII mRNA than PC3 cells.

Our results demonstrate that tumor suppressive activity of INHA is altered during the transition from AD to AI PCa. It provides the first functional evidence which suggests that loss in the tumor suppressive activity of INHA in different stages of prostate disease may be due to loss in TGF $\beta$ RIII expression.

*Financial Support:* These studies were supported by Australian National Health and Medical Research Council program grant and a Post-doctoral training award by United States Department of Defense [PB, Grant#: PC060112].

## APPENDIX 7: LORNE CANCER ABSTRACT

### **Tumor suppressive activity of inhibin- $\alpha$ subunit is altered during the transition from androgen-dependent to androgen-independent prostate cancer**

Preetika Balanathan<sup>1</sup>, Elizabeth D Williams<sup>2</sup>, Hong Wang<sup>1</sup>, Marc G Achen<sup>3</sup>, Steven A Stacker<sup>3</sup>, Gail Risbridger<sup>1</sup>. <sup>1</sup>Centre for Urological Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. <sup>2</sup>Centre for Cancer Research, Monash Institute of Medical Research, Monash University, Melbourne, Victoria, Australia. <sup>3</sup>Ludwig Institute for Cancer Research, Post Office Box 2008, Royal Melbourne Hospital, Melbourne, Victoria, Australia.

The transition from androgen-dependent (AD) to androgen-independent (AI) disease is a key event in prostate cancer (PCa) progression and Inhibin- $\alpha$  subunit (INHA) has been proposed to have a tumor suppressive and pro-metastatic role during different stages of the disease. Recently, loss of TGF $\beta$  receptor RIII (TGF $\beta$ RIII), a receptor for inhibin has been proposed to be an explanation for the different activities of INHA in PCa.

The AD, LNCaP and AI PC3 cell lines were evaluated for their sensitive to INHA expression in the presence of endogenous levels of TGF $\beta$ RIII. Over-expression of INHA in AD LNCaP cells decreased cell proliferation, migration and reduced tumor growth supporting the role of INHA as a tumor suppressor. In contrast, over-expression of INHA in AI PC3 cells increased cell proliferation, migration, tumor growth and metastasis supporting the loss of tumor suppressive activity/gain in metastatic properties for INHA in AI stage of the disease. The shift in the tumor suppressive activity of INHA was further evident by increase in lymph node metastasis in the INHA over-expressing PC3 tumors which was accompanied by an elevation of lymphatic vessel density, tumor cell invasion into lymphatics and up-regulation of VEGF-C. Consistent with other studies our work revealed that LNCaP cells expressed significantly more TGF $\beta$ RIII mRNA than PC3 cells. Analysis of human PCa specimens showed that INHA expression cannot be used to determine lymph node status in PCa patients. However, increase in INHA expression by normal epithelium and prostate intraepithelial neoplasia (PIN) regions of the tumors in patients with lymph node metastasis suggests that INHA may have a paracrine role that, directly or indirectly, promotes the spread of cancer cells from the primary prostate tumor to the lymph nodes.

Our results demonstrate that tumor suppressive activity of INHA is altered during the transition from AD to AI PCa. It provides the first functional evidence which suggests that loss in the tumor suppressive activity of INHA in PCa progression may be due to loss in TGF $\beta$ RIII expression.

## APPENDIX 8: GSK GRANT



3 October 2007  
Ref: DR07/091

1061 Mountain Highway  
Boronia Victoria 3155  
PO Box 168 Boronia 3155  
Australia  
Tel. +61 03 9721 6000  
Fax. +61 03 9729 5319  
www.gsk.com.au

Professor Gail Risbridger  
Director  
Centre for Urological Research  
Monash Institute of Medical Research  
Monash University  
27-31 Wright St  
Clayton VIC 3168

*Marge*  
*Roco will need to*  
*sign this plse.*  
*+ Prue*

Dear Professor Risbridger,

### GSKA Post Graduate Support Grant – 2007 Application Round


On behalf of Dr Michael Elliott, Vice President & Area Medical Director, Australasia & Asia-Pacific, I am delighted to inform you that your application for the GSKA Post Graduate Support Grant has been successful. You may be interested to learn that your application was one of 9 selected for funding from a pool of 319 applications.

Please find enclosed duplicate copies of the Research Agreement for the abovementioned grant. The award will be \$25,000 over two years.

#### For your action:


1. Arrange for both copies of the enclosed Research Agreement to be executed by authorised representatives for your institution.
2. Retain one copy of the Research Agreement for your records and return the second executed copy to myself using the enclosed self addressed envelope, **by no later than Friday 2 November 2007.**
3. Institution to raise a tax invoice for the amount of \$16,500 (incl GST) being for the first payment, and forward to me **by no later Friday 2 November 2007.** (Please include Institute's banking details to enable GSK to make payments by Electronic Funds Transfer).

We would also like to publish the 2007 grant winners details on our website and possibly through a media release. No project details would be disclosed other than the project title. Could you please advise us if you would prefer that we do not publish this information.



I would like to take this opportunity to congratulate you and Preetika Balanathan on your successful application. GSK looks forward to hearing about the progress on your exciting project.

Yours sincerely,  
**GlaxoSmithKline Australia Pty Ltd**



**Ashley Bates, PhD**  
**Head of R&D Alliances Aust/NZ**

Encl.

cc: Preetika Balanathan

## APPENDIX 9: ANZ GRANT



ANZ Trustees  
Philanthropy Partners  
Level 4, 100 Queen Street, Melbourne Vic 3000  
GPO Box 389D, Melbourne Vic 3001  
Telephone 03 9273 6799  
Facsimile 03 9273 6354

15 October 2007

Dr Preetika Balanathan  
Monash University  
Monash Institute of Medical Research  
27-31 Wright Street  
Clayton Vic 3168

PROCESSED

Dear Dr Balanathan

Project name: **Dr Preetika Balanathan, Professor Gail Risbridger - Dual or multi-functionality of inhibin-a subunit in prostate cancer progression**  
Organisation: **Monash University**  
Application date: **29 June 2007**  
Reference number: **CT 9012**

On behalf of the Trustees of **Medical Research & Technology in Victoria - The William Buckland Foundation**, I am pleased to advise that your organisation has been granted an amount of **\$15,000** to be used towards the project described in your application.

With this letter we have included:

- Grant conditions
- Advice on acknowledging The William Buckland Foundation
- Reporting format

The grant is to be expended in Victoria only. Banking the enclosed cheque indicates acceptance of the grant for the purpose of the project outlined in your application and the grant conditions attached to this letter.

As set out in condition 7, your organisation is required to submit annual progress reports and a final report (or only a final report in the case of a one year project) on the project.

The Trustees wish you every success with the project and look forward to watching the progress of your project.

Yours sincerely

  
Trisha Broadbridge  
Manager, Philanthropy Partners, ANZ Trustees Limited

Enc

## APPENDIX 10: TRAVEL SCHOLARSHIP



### KEYSTONE SYMPOSIA

Connecting the Scientific Community

28 January, 2008

Preetika Balanathan  
Centre for Urological Research  
Monash Institute of Medical Research, Monash University  
27-31 Wright Street  
Melbourne, Victoria 3168 Australia

Dear Preetika Balanathan:

Congratulations on winning a scholarship for up to \$1000.00 for reimbursement of costs associated with travel to the Keystone Symposia meeting TGF- $\beta$  Family in Homeostasis and Disease to be held at Eldorado Hotel & Spa, Santa Fe, New Mexico on February 3 - 8, 2008.

Immediately upon your return home, mail your original registration, air travel, ground transportation and conference lodging receipts totaling up to \$1000 to me using the pre-addressed envelope. Remember, we do not reimburse meals, incidentals or recreational expenses.

I recommend that you don't use "express check-out" upon departure from the hotel. It is very difficult to get an original receipt after you have left. It would be best to pick up your itemized receipt before leaving the hotel. If you are sharing accommodations, ask for a separate receipt with your name and portion of the hotel bill.

We can accept copies of the receipts *only* if we are reimbursing your institute, in which case, please include any identifying information; i.e. name to whom the check should be made payable, department name or account number, etc. We can also accept print outs from air travel arrangements made on the internet if they include your name, departure, destination, dates and the cost. All receipts submitted should include this information. Other allowable expenses are meeting registration, ground transportation from the airport to the meeting, car rental or mileage if using a personal vehicle. We will not reimburse credit card receipts or statements so be sure to obtain all original receipts at time service is rendered. If a wire transfer is requested a \$20.00 USD fee will be deducted from the award. The cost incurred by us is \$40.00 USD.

Reimbursement checks will be processed as soon as *all* receipts from *all* scholarship winners have been received. **If I do not hear from you nor receive your receipts in our office on or before 21 February 2008 your scholarship award will be forfeited. Please do not delay in sending your receipts; it will be unfair to the others.**

When sending your receipts please go into your account on our website [www.keystonesymposia.org](http://www.keystonesymposia.org) and in "Student/Postdoc Scholarship Application" under "Award Information" note the mailing address to which you would like us to mail your reimbursement check to. You can check the status of your receipts in your account as well, e.g. "Not Received", "Received", "Processed", "Payment Sent".

Please do not call our office to check on the status of the receipts. You can check it on our website in your account in "Student/Postdoc Scholarship Application" under "Receipt Status", e.g. "Not Received", "Received", "Processed", "Payment Sent". This information gets updated regularly.

Please also note that reimbursement can be issued only in your name or in the name of your institute.

If you have any questions, you can contact me at 1-800-253-0685 ext.140 or 970-262-1230 ext.140 or at [ksenias@keystonesymposia.org](mailto:ksenias@keystonesymposia.org).

Enjoy the conference!

Ksenia Shambarger  
Scholarships

Program Development & Implementation • 221 Summit Place #272 • PO Box 1630 • Silverthorne, CO 80498  
800-253-0685 • 970-262-1230 • 970-262-0311 (fax) • [programs@keystonesymposia.org](mailto:programs@keystonesymposia.org) • [www.keystonesymposia.org](http://www.keystonesymposia.org)